395-208
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A/RE Issue

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PATENT
Attorney Docket No.: KM39091-70

TO: Assistant Commissioner of Patents
Washington, D.C. 20231
Box: Reissue Application

S I R :

With reference to the filing in the United States Patent and Trademark Office of an application for a Reissue patent in the name(s) of: Laurette NACAMULLI et al. entitled: **RATE MEASUREMENTS OF BIOMOLECULAR REACTIONS USING ELECTROCHEMILUMINENSCENCE.**

_X_ This is an application of a small entity under 37 CFR 1.9(f) and the amounts shown in parentheses below have been employed in calculating the fee.

_X_ Small Entity Verified Statement was filed in the parent application (08/347,984) and is still proper.

The following are enclosed:

_X_ Specification (copy of U. S. Patent No. 5,527,710 and originally filed Specification)

The original Letters Patent are presently inaccessible. However, they will be submitted if and when they become accessible.

_X_ _70_ Claim(s) (including 36 claims from the original patent and _34_ claims added in this Reissue Application, said added claims including _4_ independent claims and _30_ dependent claims).

____ This application contains a multiple dependent claim.

_X_ Reissue Declaration and Power of Attorney (executed by two of three inventors)

____ ___ Sheet(s) of Drawings. No changes have been made in the drawings upon which the original patent was issued, therefore in accordance with 37 C.F.R. §1.174, enclosed is a copy of the printed drawings of the patent comprising __ sheets of drawings.

_X_ Our check No. _1354_ for $933.00, calculated as follows:

Basic Fee - $790.00 ($395.00)	$395.00
X Total Number of Independent Claims over original patent _4_ at $82.00 ($41.00) each	$164.00
Total Number of Claims in excess of 20 and over those of original patent _34_ at $22.00 ($11.00) each	$374.00
Total Filing Fee	$933.00

Please charge any additional fees required for the filing of this application or credit any overpayment to Deposit Account No. 50-0297.

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Date of Deposit June 17, 1998
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(Signature of person mailing paper or fee)

Respectfully submitted,

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y421090.jebkm3909170

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PATENT
KM39091-70

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Laurette NACAMULLI et al.

Filed: Herewith

For: Application to Reissue U.S. Patent No. 5,527,710 granted June 18, 1996

Entitled: **RATE MEASUREMENT OF BIOMOLECULAR REACTIONS USING ELECTROCHEMILUMINESCENCE**

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OFFER TO SURRENDER

Assistant Commissioner for Patents
Washington, D.C. 20231
Box: Reissue Application

S I R :

The undersigned officer of the Assignee in the accompanying reissue application to reissue U.S. Patent No. 5,527,710, granted June 18, 1996, which is owned by the Assignee, as it is indicated by the Title Report which has been requested herewith, hereby offers to surrender said Letters Patent.

Dated: 6-12-98

Richard Massey
President
IGEN International, Inc.

PATENT
KM39091-70

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Laurette NACAMULLI et al.

Filed: Herewith

For: Application to Reissue U.S. Patent No. 5,527,710 granted June 18, 1996

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ASSENT BY ASSIGNEE

Assistant Commissioner for Patents
Washington, D.C. 20231
Box: Reissue Application

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The undersigned representative of the Assignee in the accompanying reissue application to reissue U.S. Patent No. 5,527,710, granted June 18, 1996, which is owned by the Assignee, as it is indicated by the Title Report which has been requested herewith, hereby assents to the filing of the reissue application.

Dated: 6 12 98

Richard Massey
President
IGEN International, Inc.

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RATE MEASUREMENTS OF BIOMOLECULAR REACTIONS USING ELECTROCHEMILUMINESCENCE

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to analytical methods and systems for measuring the rate of biomolecular reactions. More particularly, the invention has to do with the use of electrochemiluminescence ("ECL") to monitor in real time the progress of a biomolecular reaction. The method can be used to monitor the progress of affinity binding reactions and, as such, can be used in antibody-antigen binding rate measurements, among others. Th method also can be used for the diagnostic determination of a enzyme activity or concentration and for other rate measuremen as will be apparent to those skilled in the art.

Description of Related Art

There are various known methods for measuring the progre of biomolecular reactions and the present invention provides new method of monitoring the rates of such reactions. T progress of enzymatic reactions, for example, has been monitore by spectrophotometry and fluorescence. These methods and others are used in modern laboratories and have been used by applicants to obtain reference data for the development of the new analytical technique of the present invention.

Antigen-antibody reaction rates can be measured using a technology called real-time biospecific interaction analysis which uses surface plasmon resonance to detect biomolecular interactions. The method was reported to be a valuable supplement to conventional methods of investigation in an article entitled "Label-Free Biosensor Technology Visualizes Biomolecular Interactions in Real Time", *Biosensors & Bioelectronics*, Vol. 8, No. 2, Products and Innovations, pp. xi-xiv. The use of surface plasmon resonance is also discussed by Sjolander, S. and Urbaniczky, C. in "Integrated Fluid Handling System for Biomolecular Interaction Analysis", *Analytical Chemistry* 1991, Vol. 63, pp. 2338-2345. According to the method, the kinetics for biomolecular interactions between an antigen and an antibody can be followed directl without labeling. The method is useful for detecting, in sit low concentrations of biochemically active molecules having hi molecular weight.

A general procedure for the determination of the dissoc - tion constant (K_D) of antigen-antibody equilibria in solution ; reported by Friguet, B., et al., in "Measurements of the T e Affinity Constant in Solution of Antigen-Antibody Complexes / Enzyme-Linked Immunosorbent Assay", *Journal of Immunologi Methods* 1985, Vol. 77, pp. 305-319. The method employs a class - cal indirect ELISA and is reported to permit the detection of very small concentrations of antibody and the determination of K_D values as small as 10^{-9}M.

The method and system of the present invention employ elec-

trochemiluminescence which has been used heretofore in analytical methods for the qualitative and quantitative analysis of chemical moieties. In United States Patent No. 5,310,687, for example, a chemical moiety is disclosed which comprises a chemical, biochemical or biological substance attached to one or more electrochemiluminescent organometallic compounds. Methods are disclosed for detecting low concentrations of the chemical moiety using chemiluminescent, electrochemiluminescent and photo-luminescent means. Compounds are disclosed which are useful for labeling substances of interest with ruthenium-containing and osmium-containing labels or other electrochemiluminescent labels. The labeled substances are useful in methods for detecting and quantifying analytes of interest in binding assays and competitive binding assays.

We have now discovered a method and system of employi electrochemiluminescence to monitor the progress of biomolecul reactions and the method can be employed in diagnostic kits f clinical use, research laboratories, and the like. The met employs commercially available equipment and provides a hig accurate means for diagnostic determination of an enzyme activ or concentration. The method also provides a means to measu antibody-antigen binding rates and it is useful for screening f high binding rate antibodies. In one embodiment, a method has been derived for measuring the rates of antibody binding to carcinoma embryonic antigen. In another embodiment, a method has been derived to determine lactate dehydrogenase for clinical

applications.

SUMMARY OF THE INVENTION

A biomolecular reaction which is to be monitored according to the present invention must be carried out using a luminophore under reaction conditions which will relate the concentration of a reactant or a product of the reaction to the ECL intensity. The reagents employed in the reaction, therefore, will include a reaction partner which reacts with the reactant and participates with the luminophore to cause the emission of ECL. In some embodiments, it is the reaction product of the reaction partner which participates with the luminophore to cause the emission of ECL. The method of the invention also requires the modulation and measurement of the ECL intensity of the biomolecular reactio and the demodulation of the intensity measurement.

The biomolecular reaction is carried out in an electrochem cal cell and a series of electrical pulses are applied at preselected potential and at preselected constant intervals time and constant duration to modulate the ECL output. The tensity of the resulting luminescence is measured at the s intervals to provide a timed series of values called reaction progress (P). The same experiment is repeated, except that it allowed to go to completion before ECL intensity is measured pulsing and measuring luminescence under the same conditions to provide a timed series of values called reaction complete (C). The same experiment is repeated a third time in the absence of the reaction partner and the ECL intensity is measured at the

same intervals to provide a timed series of values called blank (background reaction) (B).

The time course, concentration vs. time, of the reaction is determined by demodulating the intensity measurements. This is accomplished by subtracting the blank (B) from the reaction in progress (P) and dividing by the difference of the reaction complete (C) less the blank (B). Accordingly, the enzymatic reaction (P) is normalized (N) by the following formula:

(I) $$N = \frac{P - B}{C - B}$$

The time course is compared to a known standard to determine concentration over time and the reaction rate can be determined at any point in time by taking the first derivative (tangential slope) at that point on the concentration vs. time curve.

The enzyme rate measurement method of the invention requires an enzymatic reaction which produces or consumes a substance which is ECL active. As the reaction progresses the ECL intensity will vary with the concentration of the ECL active substance. A luminophore and the ECL active substance (or the substance which produces the ECL active substance) are mixed with the other reactants. The enzyme is added last and the reaction is allowed to proceed in an electrochemical cell. A series of electrical pulses is applied, as explained above, and the ECL intensity is measured and demodulated to obtain the time course of the reaction.

In the measurement of binding reaction rates, for example, antibody-antigen binding rates, two reagents are prepared prior to the binding event. A luminophore, such as Ru(2,2'-bipyridine)$_3^{2+}$ (sometimes abbreviated herein as "Ru(bpy)$_3^{2+}$" and the bipyridine ligand itself is sometimes abbreviated herein as "bpy"), is attached to the antibody whose binding rate is to be determined, and the antigen (the reaction partner) is attached to a magnetic bead. An ORIGEN® Analyzer available from Igen, Inc., 1530 East Jefferson Street, Rockville, MD 20852, U.S.A., can be used to dispense samples containing the magnetic beads and conduct the analysis. The samples are drawn into the electrochemical flow cell of the Analyzer and the antigen coated magnetic beads are deposited uniformly onto the working electrode from the flow stream by placing the magnet directly below. The binding event is initiated and progressed by continuously drawing labeled antibody through the electrochemical flow cell of the analyzer. As the binding event proceeds the ECL active label binds to the magnetic bead. A series of electrical pulses are applied as described above. A rise in the ECL occurs as the binding proceeds and indicates reaction progress. The ECL intensity is then demodulated to obtain the time course of the reaction.

The electrochemiluminescent labels used according to the invention are sensitive, non hazardous and inexpensive, and they can be used in a wide variety of applications.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The time course of a biomolecular reaction is determined

according to the present invention by forming a first reagent mixture containing a reactant, a luminophore and a reaction partner. The reactant reacts with the reaction partner, and the luminophore participates with the reaction partner to emit electrochemiluminescence upon exposure of the reagent mixture to electrical energy. In some embodiments of the invention, the reaction product of the reaction partner, rather than the reaction partner itself, participates with the luminophore to emit electrochemiluminescence. A series of electrical pulses is applied to the first reagent mixture at a preselected potential and at preselected intervals of time and duration, and the electrochemiluminescence is measured at the same intervals to obtain a value for each interval.

A second reagent mixture is formed which is the same as th first reagent mixture. The reagents of the second reagent mi: ture are allowed to react until the reaction is complete and th the mixture is exposed to a series of electrical pulses at t same potential, intervals of time and duration as was the fir reagent mixture. The electrochemiluminescence also is measur at the same intervals as for the first reagent mixture to obta a value for each interval.

A third reagent mixture is formed which is the same as th first reagent mixture except that it does not contain the reaction partner. A series of electrical pulses is applied to the third reagent mixture at the same potential, intervals of time and duration was the first reagent mixture. The electrochemilu-

minescence is measured at the same intervals as for the first reagent mixture to obtain a value for each interval.

The value obtained for the first interval for the third reagent mixture is subtracted from the value obtained for the first interval for the first reagent mixture to obtain a first difference. The value obtained for the first interval for the third reagent mixture also is subtracted from the value obtained for the first interval for the second reagent mixture to obtain a second difference. The first difference is divided by the second difference to obtain a normalized value for the first interval. The normalized value then is calculated in the same way for each successive interval to obtain a series of normalized values which can be plotted to illustrate the time course (concentration vs. time) graphically. Other mathematical operations can be performed on the data as will be apparent to those skilled in the art. For example, one can take the first derivative at any point on the normalized value curve to determine the rate of the reaction at that point.

The system of the invention comprises a first reagent mixture containing a reactant, a luminophore and a reaction partner. The reactant reacts with the reaction partner, and the luminophore participates with the reaction partner, or the reaction product of the reaction partner, to emit electrochemiluminescence upon exposure of the reagent mixture to electrical energy. The system further comprises a second reagent mixture which is the same as the first reagent mixture except that the reagents have

been allowed to react and therefore it comprises reacted reagents. A third reagent mixture which is the same as the first reagent mixture except that it does not contain the reaction partner is also provided with the system. Finally, the system is provided with a means for separately exposing each of the first, second and third reagent mixtures to a series of electrical pulses at a preselected potential and at preselected intervals of time and duration, and a means for measuring the electrochemiluminescence at the same intervals.

The method of the invention is conducted in an apparatus provided with an electrode, such as an electrochemical cell. The biomolecular reaction which is monitored according to the invention is carried out in the apparatus using a luminophore under reaction conditions which will relate the concentration of a reactant, a reaction partner or the reaction product of the reaction partner to the ECL intensity, and the reaction partner is reagent which reacts with the reactant and which participates (or its reaction product participates) with the luminophore to cause the emission of ECL.

The ECL intensity of the biomolecular reaction is modulated by means of an input potential applied to the electrode. The input potential induces the luminophore to emit a measurable ECL output by creating an excited state of the luminophore which luminesces at wavelengths between about 200 nanometers ("nm") and 900 nm at ambient temperatures. The input potential is incremented with time (e.g., the RAMP method), and the ECL output is

detected as the response to the voltage change. The ECL peak occurs at the potential of the electrochemical reaction (peak potential) which is driven by the input potential.

Another way of generating ECL is by stepping the input voltage at or higher than the peak potential. The ECL then is observed as a sharp peak that decays with time.

According to the method of the invention a series of short pulses is applied to the electrode, also at a slightly higher voltage than the peak potential. The result is a series of ECL peaks corresponding to the individual voltage pulses. In this manner, a "sampling" of the ECL signal is obtained at constant intervals in time and the progression of a reaction with time can be followed. The intensity of this modulated ECL signal is measured at each time interval and the measurements are then demodulated to obtain the time course of the reaction.

The ECL reaction is slowed down by the method of the invention by using narrow voltage pulses and the decay rate due to t biomolecular reaction is compared to the decay rate due to t ECL reaction. (In an ECL reaction where no enzymatic reaction going on, one long pulse at a high value will provide an E intensity which will decay over time.) In the time interva between pulses, new material diffuses to the electrode and i ready to react when the next voltage pulse comes, therefore each ECL peak is only slightly lower than the previous one.

Several parameters can be controlled such as the pulse potential and duration, the rest potential (i.e., the potential

during the interval between pulses) and the time between each pulse as well as the concentration of the reactants to provide for better conditions for rate measurements for each particular biomolecular reaction, as will be apparent to those skilled in the art.

When the series of electrical pulses are applied at a preselected potential and at preselected constant intervals of time and constant duration to modulate the ECL output, the intensity of the resulting luminescence is measured at the same intervals to provide a timed series of values called reaction in progress (P). The same experiment is repeated, except that it is allowed to go to completion before ECL intensity is measured by pulsing and measuring luminescence under the same conditions to provide a timed series of values called reaction complete (C). The same experiment is repeated a third time in the absence of the reaction partner and the ECL intensity is measured at the same intervals to provide a timed series of values called blank (B).

The time course, concentration vs. time, of the reaction is determined by demodulating the intensity measurements. This is accomplished by subtracting the blank (B) from the reaction progress (P) and dividing by the difference of the reaction complete (C) less the blank (B). Accordingly, the enzymatic reaction (P) is normalized (N) by the following formula:

$$N = \frac{P - B}{C - B} \qquad (I)$$

The time course is compared to a known standard to determine concentration over time and the reaction rate can be determined at any point in time by taking the first derivative (tangential slope) at that point on the concentration vs. time curve.

The method of the present invention as it applies to enzymatic reactions is generally suited to measuring the rate of oxido-reductase reactions. Oxido reductases catalyze oxidation reduction reactions and are classified in six categories as acting on (1) $\rangle$CH-OH; (2) $\rangle$C=O; (3) $\rangle$C=CH-; (4) $\rangle$CH-NH_2; (5) $\rangle$CH-NH-; and (6) NADH; NADPH.

The category of oxido reductases that is particularly suited to the method of the present invention is the dehydrogenases. As a group, dehydrogenases require for their activity a cofactor. A cofactor is a nonprotein component and it may be a metal ion or an organic molecule called a coenzyme. Suitable cofactors for dehydrogenases (as well as oxidases and other enzymes) are described in the literature and are well known in the art. Typical coenzymes for dehydrogenases are, for example, nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH).

Generally, a reaction catalyzed by a dehydrogenase, using NADH as an example, can be described as follows:

$$\text{reduced substrate} + NAD^+ = \text{oxidized substrate} + NADH$$

and the reaction may proceed in either direction. The substrate is the molecule on which the enzyme exerts catalytic action.

Examples of reduced substrates include isocitrate, ethanol, lactate, malate, and glucose-6-phosphate.

In accordance with the enzyme rate measurement method of the present invention, substances (cofactors) are employed which are ECL active (i.e., the reaction partner) and which co-react with the reactant of an enzymatic reaction. Alternatively, the ECL active substance can be the reaction product of the reaction partner. As noted above, the cofactors include NADH and NADPH and the respective oxidized forms thereof, NAD^+ and $NADP^+$, which are particularly suitable for use with dehydrogenases, and hydrogen peroxide (H_2O_2) which is particularly suitable for use with oxidases.

The enzymatic reaction must produce or consume the ECL active substance. As the process occurs, the substance is used for ECL, which relates the concentration of substances to the E intensity. For example, NADH produced in the following reacti is used to measure the enzyme reaction rate for glucose-phosphate dehydrogenase:

(1) glucose-6-phosphate + NAD^+ = 6-phosphogluconate + NA

(2) NADH + $Ru(bpy)_3^{2+}$ = ECL

wherein reaction (1) is carried out in the presence of glucose-phosphate dehydrogenase and $Ru(bpy)_3^{2+}$. As NADH is produced it reacts with $Ru(bpy)_3^{2+}$ according to reaction (2), when electrical pulses are applied, to produce ECL. The intensity of the ECL will increase with an increased rate of production of NADH and it will decrease with a decreased rate of production of NADH.

Alternatively, NADH consumed in the following reaction is used to measure the enzyme reaction rate for lactate dehydrogenase (LDH):

(3) pyruvate + NADH = NAD^+ + lactate

(4) NADH + $Ru(bpy)_3^{2+}$ = ECL

Wherein reaction (3) is carried out in the presence of LDH and $Ru(bpy)_3^{2+}$. As NADH is consumed it reacts less with $Ru(bpy)_3^{2+}$, according to reaction (4), when electrical pulses are applied, to produce ECL. The intensity of the ECL will decrease with an increased rate of consumption of NADH and it will increase with a decreased rate of consumption of NADH.

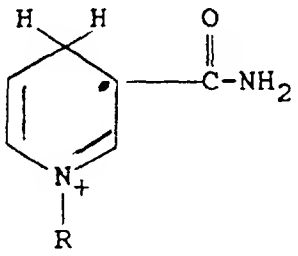
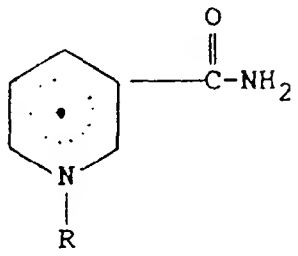
NADH is a particularly suitable coreactant for use in accordance with the enzyme rate measurement method of the presen invention because it participates in numerous enzymatic reac tions. The substrate is converted to products by the enzym and, in the process, NADH is converted to NAD^+ or vice versa d pending upon the reaction. As NADH is produced (or consumed) participates with the luminophore to yield ECL. The intensity the light is proportional to the concentration of NADH at eac time point. The change in NADH concentration is related to th activity of the enzyme catalyzing the reaction. The signal is plotted vs. time after point-by-point subtraction of the background and normalizing the signal of the luminophore with the initial (or final) concentration of NADH to obtain a time course.

The reaction mechanism between the $Ru(bpy)_3^{2+}$ and NADH as

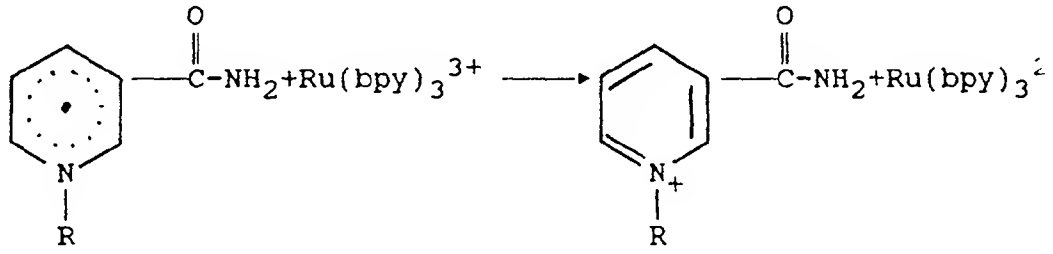
the coreactant that ultimately produces electrochemiluminescence involves a first step of oxidizing $Ru(bpy)_3^{2+}$ at the electrode according to (1) as follows:

(1) $Ru(bpy)_3^{2+} \longrightarrow Ru(bpy)_3^{3+} + e^-$.

NADH is also oxidized at the electrode followed by a proton loss that produces the strong reducing agent, NAD radical according to (2) as follows:

(2) NADH $\xrightarrow[\text{oxidation}]{-e^-}$  $\xrightarrow{-H^+}$ 

Next the NAD radical reacts with $Ru(bpy)_3^{3+}$ in a homogeneou reaction (3). The energy transfer is sufficient to raise t ruthenium complex to its excited state as follows:

(3) 

Upon decay to the ground state the tag molecule emits detectable light at 620nm according to reaction (4) as follows:

(4) $Ru(bpy)_3^{2+*} \longrightarrow Ru(bpy)_3^{2+} + h\nu$

The enzyme rate measurement method of the invention is conducted by mixing all of the reagents in a sample tube, and the enzyme is added last. Upon the addition of the enzyme, the sample is drawn into the electrochemical cell of an ORIGEN® Analyzer. A series of electrical pulses are applied at constant intervals of time and constant duration. (The ORIGEN® Analyzer generates a square wave, but triangle or sine waves also can be used.) The resulting luminescence from the luminophore is measured and indicates the amount of products formed as the enzymatic reaction progresses.

The method of the present invention as it applies to binding reactions can be used to monitor reactions such as those listed below:

antibody-antigen, such as CEA to anti-CEA;

ligand-receptor, such as a hormone binding to its receptor

avidin-biotin;

base pairing, such as with DNA hybridization reactions;

lecitins-carbohydrates; and

enzyme-inhibitor.

In the measurement of binding reaction rates, two reagen are prepared prior to the binding event. When an antibod antigen reaction is involved, for example, the luminophore i attached to the antibody (the antibody being the reactant) whose binding rate is to be determined, and the antigen (the reaction partner) is attached to a magnetic bead. The ORIGEN® Analyzer can be used to dispense samples containing the magnetic beads and

conduct the analysis. The samples are drawn into the electrochemical flow cell of the Analyzer and the antigen coated magnetic beads are deposited uniformly onto the working electrode from the flow stream by placing the magnet directly below. The binding event is initiated and progressed by continuously drawing labeled antibody through the electrochemical flow cell of the analyzer. As the binding event proceeds the ECL active label binds to the magnetic bead. A series of electrical pulses are applied as described above. A rise in the ECL occurs as the binding proceeds and indicates reaction progress. The ECL intensity is then demodulated to obtain the time course of the reaction.

Luminophores which can be used in accordance with th present invention fall into two classes, namely, organic co pounds and inorganic compounds. The organic compounds incl fluorescent or phosphorescent polyaromatic hydrocarbons, such s rubrene, 9,10-diphenylanthracene, phthalocynanines, and phen - threne. The inorganic compounds include fluorescent or phosp - rescent transition metal chelates such as ruthenium tr - bipyridine, osmium tris-bipyridine, platinium diphosphona , $Mo_6Cl_{12}^{2-}$; organometallic compounds; rare earth chelates such s terbium thenoyltrifluoroaectonate; europium dibenzoylmethide; a i main group chelates such as silicon phthalocyanine. Particularly useful luminophores are Ru-containing and Os-containing compounds.

The luminophores which are disclosed in U. S. Patent No.

5,310,687 can be used as luminophores according to the present invention and, among those disclosed, ruthenium complexes such as Ru(2,2′-bipyridine)$_3^{2+}$ are preferred.

The particular labels with which the present invention is concerned are electrochemiluminescent. They can often be excited to a luminescent state without their oxidation or reduction by exposing the compounds to electromagnetic radiation or to a chemical energy source such as that created by typical oxalate-H_2O_2 systems. In addition, luminescence of these compounds can be induced by electrochemical methods which do entail their oxidation and reduction. The method of the present invention has to do with exciting these labels with electrical pulses.

Extensive work has been reported on methods for detectin Ru(2,2′-bipyridine)$_3^{2+}$ using photoluminescent, chemiluminescent and electrochemiluminescent means: Rubenstein and Bard (1981 "Electrogenerated Chemiluminescence. 37. Aqueous Ecl Syste based on Ru(2,2′-bipyridine)$_2^{2+}$ and Oxalate or Organic Acids", Am. Chem. Soc. 103, pp 512-516; and White and Bard (1982), "El trogenerated Chemilluminescence. 41. Electrogenerated Chemil minescence and Chemilluminescence of the Ru(bpy)$_3^{2+}$-$S_2O_8^{2-}$ Syst in Acetonitrile-Water Solutions", 104, p. 6891. This work demo strates that bright orange chemiluminescence can be based on th aqueous reaction of chemically generated or electrogenerated Ru(bpy)$_3^{3+}$ with strong reductants produced as intermediates in the oxidation of oxalate ions or other organic acids. Luminescence also can be achieved in organic solvent H_2O solutions by

the reaction of electrogenerated, or chemically generated, $Ru(bpy)_3^{1+}$ with strong oxidants generated during reduction of peroxydisulfate. A third mechanism for production of electrochemiluminescence from $Ru(bpy)_3^{2+}$ involves the oscillation of an electrode potential between a potential sufficiently negative to produce $Ru(bpy)_3^{1+}$ and sufficiently positive to produce $Ru(bpy)_3^{3+}$. These three methods are called, respectively, "oxidative-reduction," "reductive-oxidation," and "the $Ru(bpy)_3^{3+/+}$ regenerative system".

The oxidative-reduction method can be performed in water, and produces an intense, efficient, stable luminescence, which is relatively insensitive to the presence of oxygen or impurities. This luminescence from $Ru(bpy)_3^{2+}$ depends upon the presence o oxalate or other organic acids such as pyruvate, lactate, malc nate, tartrate and citrate, and means of oxidatively produci $Ru(bpy)_3^{3+}$ species. This oxidation can be performed chemical by such strong oxidants as PbO_2 or a Ce (IV) salt. It can performed electrochemically by a sufficiently positive potenti applied either continuously or intermittently. Suitable ele trodes for the electrochemical oxidation of $Ru(bpy)_3^{3+}$ are, f example, Pt, purolytic graphite, and glassy carbon.

The reductive-oxidation method can be performed in partiall aqueous solutions containing an organic cosolvent such as, for example, acetonitrile. This luminescence depends upon the presence of peroxydisulfate and a means of reductively producing an excited species. The reduction can be performed electrochemi-

cally by a sufficiently negative potential applied either continuously or intermittently. A suitable electrode for the electrochemical reduction of $Ru(bpy)_3^{2+}$ is, for example, a polished glassy-carbon electrode.

The $Ru(bpy)_3^{3+/+}$ regenerative system can be performed in organic solvents such as acetonitrile or in partially aqueous systems, by pulsing an electrode potential between a potential sufficiently negative to reduce $Ru(bpy)_3^{2+}$ and a potential sufficiently positive to oxidize $Ru(bpy)_3^{2+}$. A suitable electrode for such a regenerative system is, for example, a Pt electrode. This system does not consume chemical reagents and can proceed, in principle, for an unlimited duration.

These three methods of producing luminescent Ru-containin compounds have in common the repetitive oxidation-reduction c reduction-oxidation of the Ru-containing compound. The lumine cence of solutions containing these compounds is therefore high dependent on the electric potential of the applied energy sourc and is therefore highly diagnostic of the presence of the P containing compound.

According to the present invention, a chemical moiety can employed having the formula

$$[M(P)m(L^1)n(L^2)o(L^3)p(L^4)q(L^5)r(L^6)s]t(D)u$$

wherein M is ruthenium or osmium; P is a polydentate ligand of M; L^1, L^2, L^3, L^4, L^5 and L^6 are ligands of M, each of which may be the same as or different from each other ligand; D is a substance

covalently bound to one or more of P, L^1, L^2, L^3, L^4, L^5 or L^6 through one or more amide or amine linkages; m is an integer equal to or greater than 1; each of n, o, p, q, r and s is zero or an integer; t is an integer equal to or greater than 1; u is an integer equal to or greater than 1; and P, L^1, L^2, L^3, L^4, L^5, L^6 and D are of such composition and number that the chemical moiety can be induced to emit electromagnetic radiation and the total number of bonds to M provided by the ligands of M equals the coordination number of M.

The invention also employs compounds which are particularly suitable as intermediates for attaching a luminescent ruthenium- or osmium-containing label to amino groups of chemical, biochemical and biological substances. These intermediates are thu particularly suitable for creating the chemical moieties employ according to the present invention. The intermediates are t mono- and di-N-hydroxysuccinimide esters of ruthenium or oxmi bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) a their salts; and ruthenium or osmium bis (2,2′-bipyridine) (4,4 di(chloromethyl)-2,2′-bipyridine). These compounds may be sy thesized by means known in the art.

The present invention also can employ the ruthenium-contai ing or osmium-containing chemical moieties in bonding methods fo rate determinations involving analytes of interest.

$$(A)k(D)u$$

wherein A is a compound which can be induced to repeatedly emit ECL by direct exposure to an electrochemical energy source; D is

a substance such as a nucleotide, a polynucleotide, a serum-derived antibody or a monoclonal antibody (and other substances as described later in this specification) which is attached to A; k is an integer equal to or greater than 1, and u is an integer equal to or greater than 1 comprising a) forming a reagent mixture under suitable conditions containing the chemical moiety, and b) inducing the chemical moiety to repeatedly emit ECL by applying modulated electrical energy and then demodulating the ECL in accordance with the method of the present invention.

In one embodiment of the invention M is ruthenium. In another embodiment of the invention M is osmium.

The chemical moiety must have at least one polydentate ligand of M. If the moiety has greater than one polydentate ligan the polydentate ligands may be the same or different. Polyde tate ligands include aromatic and aliphatic ligands. Suitab aromatic polydentate ligands include aromatic heterocyclic li ands. Preferred aromatic heterocyclic ligands are nitrogen-c taining, such as, for example, bipyridyl, bipyrazyl, terpyrid phenanthrolyl and porphyrins.

Suitable polydentate ligands may be unsubstituted, or su stituted by any of a large number of substituents known to t art. Suitable substituents include for example, alkyl, substi tuted alkyl, aryl, substituted aryl, aralkyl, substituted aralkyl, carboxylate, carboxaldehyde, carboxamide, cyano, amino, hydroxy, imino, hydroxycarbonyl, aminocarbonyl, amidine, guanidinium, ureide, maleimide sulfur-containing groups, phosphorus

containing groups, and the carboxylate ester of N-hydroxysuccinimide.

Additionally, at least one of L^1, L^2, L^3, L^4, L^5 and L^6 may be a polydentate aromatic heterocyclic ligand. Furthermore, at least one of these polydentate aromatic heterocyclic ligands may contain nitrogen. Suitable polydentate ligands include, but are not limited to, bipyridyl, bipyrazyl, terpyridyl, phenanthroyl, a porphyrin, substituted bipyridyl, substituted bipyrazyl, substituted terpyridyl, substituted phenanthroyl or a substituted porphyrin. These substituted polydentate ligands may be substituted with an alkyl, substituted alkyl, aryl, substituted aryl, aralkyl, substituted aralkyl, carboxylate, carboxaldehyde, carboxamide, cyano, amino, hydroxy, imino, hydroxycarbonyl, aminocarbonyl, amidine, guanidinium, ureide, maleimide, a sulfur-containing group, a phosphorus-containing group or the carboxylate ester of N-hydroxysuccinimide.

The chemical moiety can contain two bidentate ligands, each of which is bipyridyl, bipryazyl, terpyridyl, phenanthrolyl, substituted bipyridyl, substituted bipyrazyl, substituted terpyridyl or substituted phenanthrolyl.

Alternatively, the chemical moiety can contain three bidentate ligands, each of which is bipyridyl, bipyrazyl, terpyridyl, phenanthrolyl, substituted bipyridyl, substituted bipyrazyl, substituted terpyridyl or substituted phenanthrolyl. The chemical moiety may comprise ruthenium. In another embodiment of the invention, the chemical moiety comprises ruthenium, two bidentate

bipyridyl ligands and one substituted bidentate bipyridyl ligand.

In still another embodiment the chemical moiety can contain a tetradentate ligand such as a porphyrin or substituted porphyrin.

The chemical moiety may have one or more monodentate ligands, a wide variety of which are known to the art. Suitable monodentate ligands include, for example, carbon monoxide, cyanides, isocyanides, halides, and aliphatic, aromatic and heterocyclic phosphines, amines, stibines, and arsines.

Particularly preferred embodiments of the chemical moiety comprise bis(2,2′-bipyridyl) ruthenium(II) and tris(2,2′-bipyridyl) ruthenium(II).

One or more of the ligands of M can be attached to additional chemical labels, such as, for example, radioactive isotopes, fluorescent components, or additional luminescent ruthenium-osmium-containing centers.

Suitable substances (D) include many biological substances, for example, whole cells, viruses, subcellular particles, proteins, lipoproteins, glycoproteins, peptides, nucleic acids, polysaccharides, lipopolysaccharides, lipids, fatty acids, cellular metabolites, hormones, pharmacological agents, tranquilizers, barbituates, alkaloids, steroids, vitamins, amino acids and sugars. Whole cells may be animal, plant or bacterial, and may be viable or dead. Examples include plant pathogens such as fungi and nematodes. Within this application the term "subcellular particles" means subcellular organelles, membrane particles as

from disrupted cells, fragments of cell walls, ribosomes, multi-enzyme complexes, and other particles which can be derived from living organisms. Also, within this application, nucleic acids means chromosomal RNA, plasmid RNA, viral RNA and recombinant INA derived from multiple sources. Nucleic acids also include RiAs, for example messenger RiAs, ribosomal IRNAs and transfer RNAS. Polypeptides include, for example, enzymes, transport proteins, receptor proteins and structural proteins such as viral coat proteins. Preferred polypeptides are enzymes and serum-derived antibodies. Particularly preferred polypeptides are monoclonal antibodies. Hormones include, for example, insulin and T4 thyroid hormone. Pharmacological agents include, for example, cardiac glycosides. It is also within the scope of this inventic to include synthetic substances which chemically resemble biolo ical materials, such as synthetic peptides, synthetic nucle acids, and synthetic membranes, vesicles and liposomes. T foregoing is not intended to be a comprehensive list of the b logical substances suitable for use in this invention, but meant only to illustrate the wide scope of the invention.

Biological and nonbiological substances (D) are covalent bound to a ligand of M through one or more amide or amine lin ages. In the case of amide linkages, the linkages may be orient ed so that material (D) is bonded directly either to the carbonyl or to the nitrogen of the amide linkage. These chemical moieties may be ionized. If so, it is understood in the art that many different counterions will serve to neutralize the charge of

preparations of the chemical moiety. Suitable cations include for example, H^+, NH_4^+, guanidinium, Ag^+, Li^+, N^+, K^+, Mg^{2+}, and Mn^{2+}. Suitable anions include, for example, halides, OH^-, carbonate, SO_4^{2-}, hexafluorophosphate and tetrafluoroborate.

The chemical moieties also are particularly suitable as intermediates for attaching a luminescent ruthenium-containing or osmium-containing label to amino groups of chemical, biochemical and biological substances. These intermediates are thus particularly suitable for synthesizing chemical moieties according to the present invention. The intermediates are the mono- and di-N-hydroxysuccinimide esters of ruthenium and osmium 4,4′-(dicarboxy)-2,2′-bipyridyl, bis(2,2′-bipyridyl) and their salts; and ruthenium and osmium 4,4′-(dichloromethyl)-2,2′-bipyridyl bis(2,2′ bipyridyl) and their salts.

The chemical structures of these intermediates and methods of preparing them are set forth in the U. S. Patent No. 5,310,6 referenced above.

A preferred method of synthesizing the ruthenium-contain N-hydroxysuccinimide esters is to first react ruthenium dichlo bis (2,2′-bipyridine) with 2,2′-bipyridine-4, 4′-dicarboxyl acid in a hot aqueous methanol solution of sodium bicarbonat After acidification, an aqueous solution of $NAPF_6$ is added to th solution of carboxylated ruthenium compound. The isolated hexafluorophosphate salt of the ruthenium complex is then esterified by reaction with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide in dimethylformamide. Of course, many varia-

tions on the structure of the N-hydroxysuccinimide component are possible without substantially altering the usefulness of the intermediates.

The intermediates may be ionized. If so, it is understood in the art that many different counterions will serve to neutralize the charge of preparations of the intermediate and form a salt. Suitable cations for forming these salts include for example NH_4^+, guandinium, Ag^+, Li^+, Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cd^{2+}. Suitable anions for forming these salts include, for example, halides, carbonate, SO_4^{2-}, hexafluorophosphate, and tetrafluoroborate.

The intermediates are useful for labeling substances containing a free amino group capable of attacking the carboxylate ester, and thereby displacing N-hydroxysuccinimide, or of attaching the chloromethyl group, and thereby displacing chloride.

Applicants' experience with $Ru(bpy)_3^{2+}$-labeled substance indicates the advantages of using ruthenium-containing and osmium-containing compounds as chemical labels. They are stable for long periods and may be attached efficiently to a wide variety of chemical, biochemical and biological materials. The labels are safe and relatively inexpensive. They give a highly characteristic signal and do not occur in nature. Measurements based on luminescence of the labels are sensitive, fast and reproducible. There is very little interference with detection based on luminescence of these labels by such components as phosphate buffered saline, Tween® (a surfactant), liver tissue extract or serum.

Luminescence-based measurement of these labels does not destroy the sample or labeled materials and may be performed repetitively. The signal is generated repeatedly by each molecule of label, thereby enhancing the sensitivity with which these labels may be detected.

Suitable conditions for forming the reagent mixture will be known to those skilled in the art and will depend on the type of reagent mixture involved. For example, suitable conditions for an aqueous reagent mixture may include appropriate concentrations of chemical moiety and other reagents such as oxidants, pH, salt concentration and the like. For a solid sample, suitable conditions for forming a reagent mixture may include addition of a conducting liquid.

The present invention can employ osmium-containing moietie as well as ruthenium-containing moieties and the wide variety c luminescent moieties which can be made by varying the chemic structure of the ligands. Each of these variations in the met and the ligands can change the precise value of the energy inp required to create the luminescent excited state. Similarly, t wavelength of the emitted electromagnetic radiation will be de pendent upon the nature and environment of the ruthenium-contain ing or osmium-containing material. Generally, photoluminescence excitation and emission will occur with electromagnetic radiation of between about 200 nanometers and about 900 nanometers in wavelength. Chemiluminescent and electrochemiluminescent emission will generally occur with the emitted electromagnetic radiation

being between about 200 nanometers and about 900 nanometers in wavelength. The potential at which the reduction or oxidation of the chemical moiety will occur depends upon its exact chemical structure as well as factors such as the pH of the solution and the nature of the electrode used. Generally, it is well known in the art how to determine the optimal emission and excitation wavelengths in a photoluminescent system, and the optimal potential and emission wavelength of an electrochemiluminescent and chemiluminescent system.

EXAMPLES

An ORIGEN® Analyzer was used in the following experimental work. The regular operation of the instrument is configured for detecting one ECL result per sample prior to the flushing of that sample to waste. We modified the regular operation so that each tube could be analyzed individually. That is, the contents of each tube were drawn into the cell and allowed to remain there while a series of pulses was applied to the sample. Each pulse provided a data point so that a series of data points was obtained for each sample.

Typically, in the case of enzymatic reactions, the first sample to be run (reaction in progress) contained the $Ru(bpy)_3^{2}$ molecule and all the reagents for the enzymatic reaction except for the enzyme. The tube was loaded on to the carousel of the Analyzer and the Analyzer was started. (The surface of the electrode was cleaned prior to operation.)

The sample mixture, still in the tube, was vortexed. At

this point the Analyzer was programmed to stop and alert the operator to pipet in the enzyme. Once this was performed, operation of the Analyzer was resumed with the instantaneous recording of the time in seconds. With each voltage pulse generating an ECL output, a time stamp was provided. In this manner, a time course of the ECL signal was obtained.

After all the data was collected for the first tube, a second sample tube that contained the same composition as the first one followed. Except in this case the enzyme had been allowed the time to act and the reaction had reached completion. The sample was then subjected to the same voltage scheme. The results from the first tube were normalized to the results from the second tube which was the ECL output (or ECL decay) only. (Alternatively, a tube that contains NADH and $Ru(bpy)_3^{2+}$ at the sam concentrations as the first tube can be assayed for thi purpose.)

A third tube was also run which contained everything exce the substrate, for background purposes. The ECL results fr this tube were subtracted from both the in-progress tube and t completed one, prior to the numerical normalization, point b point.

The instrument sampled at a rate of one data point per 10 milliseconds ("msec"). Therefore a pulse that was 100 msec long, for example, would yield 10 samplings during that period.

A similar experimental protocol was followed for antibody-antigen rate measurements as explained in more detail below.

EXAMPLE 1

Enzymatic Reaction where NADH is generated.

Glucose-6-phosphate dehydrogenase ("G-6-PDH") catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate with NAD^+ as a coreactant that is reduced to NADH. The reaction is as follows:

$$\text{Glucose-6-phosphate} + NAD^+ \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + NADH$$

The experiment was carried out in 50 mM phosphate buffer at pH 7.5. A pH of 7.0-7.5 can be used and a carbonate buffer can be used instead of phosphate. The solution contained 0.53 g/L Triton X-100. The buffer was used both as the assay buffer and incubation buffer for the sample. The concentration of luminophore was 1E-4M $Ru(bpy)_3^{2+}$ and typical concentrations for the luminophore can be from about 1E-6M to about 1E-4M.

The sample was drawn from the tube into the electrochemical cell of the ORIGEN® Analyzer at the time recorded as zero. While in the cell compartment, the electrode was subject to a series of pulses from zero to 1800 millivolts ("mV") versus Ag/AgCl. The pulse duration was 460 milliseconds ("msec") and the rest potential was zero for 250 msec. Typical pulse rates can be from about 100 to about 500 milliseconds. The number of pulses was 20, and the number can be from about 10 to about 40.

The kinetics of the reaction were followed by monitoring the ECL output with time as NADH was generated and reacted with

$Ru(bpy)_3^{2+}$. The reaction reached about 50% of completion after about 7 minutes.

The experiment was repeated under the same conditions except that the reaction was allowed to go to completion before ECL intensity was measured.

The experiment again was repeated under the same conditions except that NAD^+ was not added to the reaction mixture.

A fourth experiment was run using the same reactants as the first run in this example except that the reaction was analyzed using a spectrophotometer. The reaction reached about 50% of completion after about 7 minutes. The spectrophotometric results were compared with the normalized curve for the method of the invention and they correlated well.

EXAMPLE 2

The Inhibition Effect of the Phosphoric Group.

The experiments of Example 1 were repeated except that the concentration of phosphate buffer was varied to demonstrate the inhibition effect of the phosphoric group. The results expressed in the time it takes to convert half the amount of substrate into products ("$t_{1/2}$") are summarized in the following table:

Phosphate buffer concentration (mM)	$t_{1/2}$ from spectrophotometer (min.)	$t_{1/2}$ from electrochemiluminescence (min.)
50	1.33	1.37
100	2.36	2.30
150	-	4.73
200	7.20	7.03

As the concentration of the buffer was increased, $t_{\frac{1}{2}}$ was increased, marking a slow down in the kinetics. The results from the two methods agreed well.

EXAMPLE 3

Enzymatic Reaction where NADH is consumed.

Lactate dehydrogenase ("LDH") catalyses the conversion of pyruvate to lactate with NADH as a coreactant which is consumed to form the oxidized form NAD^+ as follows:

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

The NADH was present in a concentration of 10^{-2}M, with 10^{-4}M $Ru(bpy)_3^{2+}$, and the same experimental protocol as was followed in Example 1 was repeated here. The electrode was pulsed twenty times at 1800 mV for 460 msec and the rest potential was zero for 250 msec. The total time course was a little over 3000 msec. The normalized results compared well with the spectrophotometric analysis.

As this reaction progresses there is less NADH to react with $Ru(bpy)_3^{2+}$ and the ECL signal goes down gradually. However due to the nature of the ECL decay, the two reactions are in competition. The decay from the overall reaction appears to be faster compared to the absorbance data because there is less NADH substrate with each pulse.

It would be instrumental if the rate of the ECL decay was reduced so that the net effect measured would be due to the enzy-

matic reaction. To that effect the parameters of the pulse and the concentrations of NADH and $Ru(bpy)_3^{2+}$ were selected to provide a more stable ECL signal. The result was a very steady ECL output over a period of three minutes.

The normal clinical range of LDH in serum is 100 to 200 U/L, which is equivalent to 1 to 2nM of the enzyme, from calculation based on the activity of the enzyme. These values are right in the range assayed and, accordingly, the test can be used to determine LDH for clinical applications.

EXAMPLE 4

Streptavidin - Biotinylated DNA.

The method of measurement is very similar to that used to measure NADH and enzyme kinetic reactions as discussed above. In the NADH/enzymatic experiments, the reaction rate is very fast therefore, the sample must be quickly aspirated just before the run begins. However, for the antibody-antigen measurements, much longer reaction time is anticipated and the experiment must be set up slightly different.

The software program which controls the ORIGEN® Analyzer was modified so that a continuous flow of antibody could be drawn across the antigen labeled beads at the electrode surface. The instrument was programmed to run the reaction from a two tube set up. The antigen-labeled beads were drawn from a first tube and captured on the electrode with the magnet. The carousel was incremented and the antibody label solution (which was in substantial excess in terms of concentration) was drawn from the second

tube across the beads at the electrode surface and the binding reaction began.

Similar to the NADH/enzyme kinetic method, it was decided to use a pulse variation of the step potential voltage waveform in these experiments such that multiple pulses were generated over an extended time interval with a clock timer incorporated to keep track of the elapsed time after each pulse was generated.

When using the repetitive pulse waveform, several parameters can be optimized for the particular reaction to be measured and these can easily be determined by those skilled in the art on the basis of the guidance provided by the present specification. The parameters include the number of pulses, the pulse width, the delay time between pulses, the step potential voltage and the rest potential.

The method of measurement was very similar to that used to measure NADH and enzymatic kinetic reactions. Three separate reactions were run, and each employed two tubes as noted above. In preparation for the experiment, we coated 280 magnetic beads with streptavidin, added them to a first tube and then added a biotinylated DNA label. A biotinylated DNA labeled calibrator was added to a second tube.

Using the terms defined in this specification, the reactions were conducted as described below:

Reaction-in-progress - Once the measurement cycle began the concentration changed over time as binding occurred and the reac-

tion completed.

Tube 1: Streptavidin coated beads

Tube 2: Biotinylated DNA labeled calibrator

Reaction-complete - The reaction was previously allowed to go to completion, so the intensity measurements were a result of the ECL decay only.

Tube 1: Streptavidin coated beads combined with biotinylated DNA label and allowed to bind completely.

Tube 2: Biotinylated DNA labeled calibrator.

Background reaction - There were no beads present, therefore the signal generated was a result of the label (background) only.

Tube 1: Assay Buffer (no beads present for binding).

Tube 2: Biotinylated DNA labeled calibrator.

After the three reactions were run, the normalized reactic curve was obtained using the formula:

(I) $$N = \frac{P-B}{C-B}$$

as explained above.

A tube-to-tube time course was then run using twenty tubes of beads and label at the same concentrations used above. They were pipetted and the run was immediately started. Therefore, the binding reaction took place in each tube, and the time of completion could be determined when the curve (ECL intensity vs. time) began to plateau. According to this time course, the reac-

tion was completed in around 6 minutes. This was a much faster completion time than that obtained by the 3-step time course method.

At this point it was suspected that the streptavidin-biotin reaction was diffusion limited when using the 3-step method. To lend more support to this conclusion, a half-life study was performed using the 3-step time course method.

The 3-step time course method was repeated using 6 different concentrations of beads: 20 ug, 4 ug, 2 ug, 1.3 ug, 1.0 ug, and 0.8 ug (per 300 uL). As would be expected, as the bead concentration was decreased the reaction completed much faster.

Subsequently, the half-life of each concentration was obtained. Since the label was in substantial excess, the reaction was pushed to follow pseudo-first order kinetics. In a first order reaction, the half-life ("$t_{\frac{1}{2}}$") is independent of concentration and is defined as:

$$t_{\frac{1}{2}}=0.693/k$$

where k is the binding constant. Therefore, the half-life should be constant regardless of concentration if the reaction is first order. In a plot of bead concentration versus half life, where the $t_{\frac{1}{2}}$ value was taken at 0.5 minutes, assuming that all of the normalized reactions should plateau at a relative value of 1.0, the half-life steadily decreased as the concentration decreased. The results are summarized below:

bead concentration (ug.)	half-life (minutes)
0.8	7.5
1.0	8
1.3	10
2	12
4	16
20	35

This steady decrease in the $t_{\frac{1}{2}}$ value is an indication that the streptavidin-biotin system is mass transfer limited at the electrode surface. This explains why the reaction took much longer to complete when using the 3-step method versus the tube-to-tube method.

Although the streptavidin-biotin reaction was diffusio limited, the ECL 3-step time course measurement method should b feasible on a system in which the binding rates are significantl slower and therefore not diffusion limited.

EXAMPLE 5

The CEA Antibody-Antigen System.

The CEA assay format used in these experiments consisted of streptavidin-coated beads bound to a biotinylated CEA antigen which is subsequently bound to a labeled CEA specific antibody. The reaction rate being measured in this system is not the streptavidin-biotin reaction but the CEA antibody-antigen reaction.

A CEA antibody referred to as 1F3 was obtained, and the ECL

3-step time course method was attempted on this system to be compared with values previously obtained on the BIAcore system using the same 1F3 antibody. (The BIAcore system employs surface plasmon resonance and it is available from Pharmacia Biosensor AB.)

The highest concentration of 1F3 label chosen to run using the 3-step method was 11 nM, which is comparable in concentration to the 10 nM low end concentration run on the BIAcore. In addition, two lower concentrations of 5.5 nM and 2.7 nM were run. The normalized profile obtained from the 11 nM 1F3 label solution had the desired shape, however it was very noisy. This is because in using the ECL method as described, both the label which binds to the beads, and the free label which does not bind (which is considered background signal) are simultaneously present a the electrode surface. At high concentrations of label, it becomes difficult to discriminate between the bound and the unboun phases, hence a smooth normalized reaction profile cannot b obtained. The normalized profiles for the 5.5 nM and 2.7 n concentrations of 1F3 antibody were much smoother, which agai supports how the contribution of the unbound label at the electrode can affect the amount of noise in the signal. Since the concentration of unbound label present at the electrode is less, it is much easier to discriminate the bound phase signal and a much smoother profile is obtained.

On the BIAcore system, a series of 1F3 label concentrations, ranging from 10 nM to 500 nM were run. The current 3-step method

on the ECL system does not allow for label concentrations much higher than 10 nM.

To obtain a comparison of the ECL 3-step method to the BIAcore method, a mathematical analysis was performed on the data obtained for the three normalized concentration curves to derive the experimental association rate constant, k_a. The k_a value for the data obtained on the BIAcore was 4.0×10^5 $M^{-1}sec^{-1}$. For the ECL data a mean ($\pm$SD)k_a value of 9.0×10^5 ($\pm 4.7 \times 10^5$) $M^{-1}sec^{-1}$ was obtained. This value is an average of the three k_a values for each concentration.

What is Claimed is:

1. A method of determining the time course of a biomolecular reaction comprising

(a) forming a first reagent mixture containing a reactant, a luminophore and a reaction partner wherein the reactant reacts with the reaction partner, and the luminophore participates with the reaction partner, or the reaction product of the reaction partner, to emit electrochemiluminescence upon exposure of the reagent mixture to electrical energy;

(b) exposing the first reagent mixture to a series of electrical pulses at a preselected potential and at preselected intervals of time and duration, and measuring the electrochemiluminescence at the same intervals to obtain a value for each interval;

(c) forming a second reagent mixture which is the same as the first reagent mixture;

(d) allowing the second reagent mixture to react until the reaction is complete and then exposing the mixture to a series of electrical pulses at the same potential, intervals of time and duration as in step (b) and measuring the electrochemiluminescence at the same intervals as in step (b) to obtain a value for each interval;

(e) forming a third reagent mixture which is the same as the first reagent mixture except that it does not contain the reaction partner;

(f) exposing the third reagent mixture to a series of electrical pulses at the same potential, intervals of time and duration as in step (b) and measuring the electrochemiluminescence at the same intervals as in step (b) to obtain a value for each interval;

(g) subtracting the value obtained for the first interval in step (f) from the value obtained for the first interval in step (b) to obtain a first difference;

(h) subtracting the value obtained for the first interval in step (f) from the value obtained for the first interval in step (d) to obtain a second difference;

(i) dividing the first difference by the second difference to obtain a normalized value for the first interval;

(j) repeating steps (g), (h) and (i) for each successive interval to obtain the normalized value for each successive interval.

2. The method of claim 1 wherein the reactant and the luminophore are combined in a chemical moiety having the formula

$$[M(P)m(L^1)n(L^2)o(L^3)p(L^4)q(L^5)r(L^6)s]t(D)u$$

wherein M is ruthenium or osmium; P is a polydentate ligand of M; L^1, L^2, L^3, L^4, L^5 and L^6 are ligands of M, each of which may be the same as or different from each other ligand; D is a substance covalently bound to one or more of P, L^1, L^2, L^3, L^4, L^5 or L^6

through one or more amide or amine linkages; m is an integer equal to or greater than 1; each of n, o, p, q, r and s is zero or an integer; t is an integer equal to or greater than 1; u is an integer equal to or greater than 1; and P, L^1, L^2, L^3, L^4, L^5, L^6 and D are of such composition and number that the chemical moiety can be induced to emit electromagnetic radiation and the total number of bonds to M provided by the ligands of M equals the coordination number of M.

3. The method of claim 1 wherein the luminophore is selected from the group consisting of fluorescent or phosphorescent polyaromatic hydrocarbons and fluorescent or phosphorescent transition metal chelates.

4. The method of claim 3 wherein the transition metal chelates are organometallic compounds.

5. The method of claim 1 wherein the luminophore is selected from the group consisting of Ru-containing and Os containing compounds.

6. The method of claim 1 wherein the luminophore is ruthenium tris-bypyridine or osmium tris-bipyridine.

7. The method of claim 1 wherein the biomolecular reaction is an enzymatic reaction, the reagent mixture contains an enzyme and the reactant is a substrate on which the enzyme exerts catalytic action, and the reaction partner is a cofactor.

8. The method of claim 7 wherein the luminophore is selected from the group consisting of fluorescent or phosphorescent polyaromatic hydrocarbons and fluorescent or phosphorescent transition metal chelates.

9. The method of claim 7 wherein the enzyme is an oxido reductase.

10. The method of claim 9 wherein the oxido reductase is a dehydrogenase.

11. The method of claim 7 wherein the cofactor is a metal ion.

12. The method of claim 7 wherein the cofactor is a coenzyme.

13. The method of claim 12 wherein the coenzyme is in its oxidized form.

14. The method of claim 1 wherein the biomolecular reaction is a binding reaction.

15. The method of claim 14 wherein the binding reaction i selected from the group consisting of antibody-antigen, ligand receptor, avidin-biotin, base pairing, lectin-carbohydrate, a enzyme-inhibitor.

16. The method of claim 14 wherein the luminophore is s lected from the group consisting of fluorescent or phosphoresce polyaromatic hydrocarbons and fluorescent or phosphorescent tra sition metal chelates.

17. The method of claim 14 wherein the reactant and th luminophore are combined in a chemical moiety having the formula

$$[M(P)m(L^1)n(L^2)o(L^3)p(L^4)q(L^5)r(L^6)s]t(D)u$$

wherein M is ruthenium or osmium; P is a polydentate ligand of M; L^1, L^2, L^3, L^4, L^5 and L^6 are ligands of M, each of which may be the same as or different from each other ligand; D is a substance

covalently bound to one or more of P, L^1, L^2, L^3, L^4, L^5 or L^6 through one or more amide or amine linkages; m is an integer equal to or greater than 1; each of n, o, p, q, r and s is zero or an integer; t is an integer equal to or greater than 1; u is an integer equal to or greater than 1; and P, L^1, L^2, L^3, L^4, L^5, L^6 and D are of such composition and number that the chemical moiety can be induced to emit electromagnetic radiation and the total number of bonds to M provided by the ligands of M equals the coordination number of M.

18. A method of determining the time course of an enzymatic reaction comprising

(a) forming a first reagent mixture containing an enzyme, a reactant, a luminophore and a reaction partner wherein the reactant reacts with the reaction partner, and the luminophore participates with the reaction partner, or the reaction product of the reaction partner, to emit electrochemiluminescence upon exposure of the reagent mixture to electrical energy;

(b) exposing the first reagent mixture to a series of electrical pulses at a preselected potential and at preselected intervals of time and duration, and measuring the electrochemiluminescence at the same intervals to obtain a value for each interval;

(c) forming a second reagent mixture which is the same as the first reagent mixture;

(d) allowing the second reagent mixture to react until the reaction is complete and then exposing the

mixture to a series of electrical pulses at the same potential, intervals of time and duration as in step (b) and measuring the electrochemiluminescence at the same intervals as in step (b) to obtain a value for each interval;

(e) forming a third reagent mixture which is the same as the first reagent mixture except that it does not contain the reaction partner;

(f) exposing the third reagent mixture to a series of electrical pulses at the same potential, intervals of time and duration as in step (b) and measuring the electrochemiluminescence at the same intervals as in step (b) to obtain a value for each interval;

(g) subtracting the value obtained for the first interval in step (f) from the value obtained for the first interval in step (b) to obtain a first difference;

(h) subtracting the value obtained for the first interval in step (f) from the value obtained for the first interval in step (d) to obtain a second difference;

(i) dividing the first difference by the second difference to obtain a normalized value for the first interval;

(j) repeating steps (g), (h) and (i) for each successive interval to obtain the normalized value for each successive interval.

19. The method of claim 18 wherein the luminophore is selected from the group consisting of fluorescent or phosphorescent polyaromatic hydrocarbons and fluorescent or phosphorescent transition metal chelates.

20. The method of claim 18 wherein the enzyme is an oxido reductase.

21. A method of determining the time course of a binding reaction comprising

(a) forming a first reagent mixture containing a reactant, a reaction partner and a luminophore, wherein the reactant reacts with the reaction partner in a reaction selected from the group consisting of antibody-antigen, ligand-receptor, avidin-biotin, base pairing, lectin-carbohydrate, and enzyme-inhibitor, and the luminophore participates with the reaction partner to emit electrochemiluminescence upon exposure of the reagent mixture to electrical energy;

(b) exposing the first reagent mixture to a series of electrical pulses at a preselected potential and at preselected intervals of time and duration, and measuring the electrochemiluminescence at the same intervals to obtain a value for each interval;

(c) forming a second reagent mixture which is the same as the first reagent mixture;

(d) allowing the second reagent mixture to react until the reaction is complete and then exposing the mixture to a series of electrical pulses at the same

potential, intervals of time and duration as in step (b) and measuring the electrochemiluminescence at the same intervals as in step (b) to obtain a value for each interval;

(e) forming a third reagent mixture which is the same as the first reagent mixture except that it does not contain the reaction partner;

(f) exposing the third reagent mixture to a series of electrical pulses at the same potential, intervals of time and duration as in step (b) and measuring the electrochemiluminescence at the same intervals as in step (b) to obtain a value for each interval;

(g) subtracting the value obtained for the first interval in step (f) from the value obtained for the first interval in step (b) to obtain a first difference;

(h) subtracting the value obtained for the first interval in step (f) from the value obtained for the first interval in step (d) to obtain a second difference;

(i) dividing the first difference by the second difference to obtain a normalized value for the first interval;

(j) repeating steps (g), (h) and (i) for each successive interval to obtain the normalized value for each successive interval.

22. The method of claim 21 wherein the reactant is attached to the luminophore to form a chemical moiety having the formula

$$[M(P)m(L^1)n(L^2)o(L^3)p(L^4)q(L^5)r(L^6)s]t(D)u$$

wherein M is ruthenium or osmium; P is a polydentate ligand of M; L^1, L^2, L^3, L^4, L^5 and L^6 are ligands of M, each of which may be the same as or different from each other ligand; D is a substance covalently bound to one or more of P, L^1, L^2, L^3, L^4, L^5 or L^6 through one or more amide or amine linkages; m is an integer equal to or greater than 1; each of n, o, p, q, r and s is zero or an integer; t is an integer equal to or greater than 1; u is an integer equal to or greater than 1; and P, L^1, L^2, L^3, L^4, L^5, L^6 and D are of such composition and number that the chemical moiety can be induced to emit electromagnetic radiation and the total number of bonds to M provided by the ligands of M equals the coordination number of M.

23. The method of claim 21 wherein the luminophore is selected from the group consisting of fluorescent or phosphorescent polyaromatic hydrocarbons and fluorescent or phosphorescent transition metal chelates.

24. A system for determining the time course of a biomolecular reaction comprising

a first reagent mixture containing as reagents a reactant, a luminophore and a reaction partner wherein the reactant reacts with the reaction partner, and the luminophore participates with the reaction partner, or the reaction product of the reaction partner, to emit electrochemiluminescence upon exposure of the

reagent mixture to electrical energy; a second reagent mixture which is the same as the first reagent mixture except that it comprises reacted reagents; and a third reagent mixture which is the same as the first reagent mixture except that it does not contain the reaction partner;

a means for separately exposing each of the first, second and third reagent mixtures to a series of electrical pulses at a preselected potential and at preselected intervals of time and duration; and a means for measuring the electrochemiluminescence at the same intervals.

25. The system of claim 24 wherein the reactant and the luminophore comprise a chemical moiety having the formula

$$[M(P)m(L^1)n(L^2)o(L^3)p(L^4)q(L^5)r(L^6)s]t(D)u$$

wherein M is ruthenium or osmium; P is a polydentate ligand of M; L^1, L^2, L^3, L^4, L^5 and L^6 are ligands of M, each of which may be the same as or different from each other ligand; D is a substance covalently bound to one or more of P, L^1, L^2, L^3, L^4, L^5 or L^6 through one or more amide or amine linkages; m is an integer equal to or greater than 1; each of n, o, p, q, r and s is zero or an integer; t is an integer equal to or greater than 1; u is an integer equal to or greater than 1; and P, L^1, L^2, L^3, L^4, L^5, L^6 and D are of such composition and number that the chemical moiety can be induced to emit electromagnetic radiation and the total number of bonds to M provided by the ligands of M equals the coordination number of M.

26. The system of claim 24 wherein the luminophore is selected from the group consisting of fluorescent or phosphorescent polyaromatic hydrocarbons and fluorescent or phosphorescent transition metal chelates.

27. The system of claim 26 wherein the transition metal chelates are organometallic compounds.

28. The system of claim 24 wherein the luminophore is selected from the group consisting of Ru-containing and Os-containing compounds.

29. The system of claim 24 wherein the luminophore is ruthenium tris-bypyridine or osmium tris-bipyridine.

30. The system of claim 24 wherein the biomolecular reaction is an enzymatic reaction, the reagent mixture contains an enzyme and the reactant is a substrate on which the enzyme exerts catalytic action, and the reaction partner is a cofactor.

31. The system of claim 30 wherein the luminophore is selected from the group consisting of fluorescent or phosphorescent polyaromatic hydrocarbons and fluorescent or phosphorescent transition metal chelates.

32. The system of claim 31 wherein the enzyme is an oxide reductase.

33. The system of claim 32 wherein the oxido reductase is a dehydrogenase.

34. The system of claim 31 wherein the cofactor is a metal ion.

35. The system of claim 31 wherein the cofactor is a coenzyme.

36. The system of claim 35 wherein the coenzyme is in its oxidized form.

RATE MEASUREMENTS OF BIOMOLECULAR
REACTIONS USING ELECTROCHEMILUMINESCENCE

ABSTRACT

The rate of a biomolecular reaction, such as an enzymatic reaction or an affinity binding reaction, is measured using electrochemiluminescence ("ECL"). The reaction is conducted in an electrochemical cell with a mixture of reagents including a luminophore which will relate the concentration of a reactant, a reaction partner or the reaction product of a reaction partner to the ECL intensity. The reaction partner is a reagent which reacts with the reactant and which participates with the luminophore (or its reaction product participates with the luminophore) to cause the emission of ECL. The ECL intensity is modulated with a series of electrical pulses which are applied to the mixture of reagents at a preselected potential and for preselected intervals of time and duration. The ECL intensity is measured at the same intervals to provide a timed series of values (P). The same experiment is repeated except that the modulation is conducted after the reaction has gone to completion to obtain a timed series of values (C). The same experiment is repeated a third time in the absence of the reaction partner to obtain a times series of values (B). The results are normalized (N) using the following formula:

(I) $$N = \frac{P-B}{C-B}$$

to obtain a series of values N which can be used to plot the time course (concentration vs. time) of the reaction.

12/1/94

NEW CLAIMS ADDED IN REISSUE APPLICATION

37. A method for determining the time course of a reaction comprising:

(a) forming a composition containing a reactant and a luminophore, wherein

(i) the reactant reacts to form a reaction product;

(ii) the luminophore is capable of being induced to electrochemiluminesce; and

(iii) the electrochemiluminescence signal emitted upon exposure of said composition to electrical energy changes as said reaction progresses; and

(b) exposing the composition to electrical energy and measuring the electrochemiluminescence at different times so as to determine the time course of the reaction.

38. The method of claim 37, wherein the reaction is a bimolecular reaction of the reactant with a reaction partner.

39. The method of claim 37, wherein the reaction is a binding reaction of the reactant with a reaction partner.

40. The method of claim 37, wherein the reaction is an enzyme catalyzed reaction.

41. The method of claim 37, wherein the reactant participates with the luminophore in the electrochemiluminescent process.

42. The method of claim 37, wherein the reaction product participates with the luminophore in the electrochemiluminescent process.

43. The method of claim 37, wherein the reactant is a cofactor.

44. The method of claim 43, wherein the cofactor is NADH.

45. The method of claim 37, wherein the reaction product is a cofactor.

46. The method of claim 45, wherein the cofactor is NADH.

47. The method of claim 37, wherein the luminophore comprises an organic luminophore.

48. The method of claim 37, wherein the luminophore comprises an organometallic luminophore.

49. The method of claim 39, wherein the reactant is an antibody and the reaction partner is an antigen.

50. The method of claim 39, wherein the reactant is attached to the luminophore and the reaction partner is attached to a magnetic bead.

51. The method of claim 37, wherein step (b) comprises exposing the composition to a series of electrical energy pulses.

52. The method of claim 37, wherein step (b) comprises measuring the electrochemiluminescence at multiple intervals of time.

53. The method of claim 37, wherein said exposing to electrical energy comprises exposing the composition to a series of electrical pulses at a preselected potential and at preselected intervals of time and duration.

54. The method of claim 37, further comprising the step of determining the concentration of the reactant in a sample.

55. The method of claim 37, wherein the luminophore is selected from the group consisting of Ru-containing and Os-containing compounds.

56. The method of claim 37, wherein the luminophore is ruthenium tris-bypyridine or osmium tris-bipyridine.

57. A method for determining the time course of a binding reaction comprising:

(a) forming a composition containing a reactant, a reaction partner and a luminophore, wherein

i) the reactant and reaction partner bind to form a complex;

ii) the luminophore is capable of being induced to electrochemiluminesce; and

iii) the luminophore is attached to said reaction partner; and

(b) exposing the composition to electrical energy and measuring the electrochemiluminescence at different times so as to determine the time course of the reaction.

58. The method of claim 57, wherein the luminophore comprises an organometallic luminophore.

59. The method of claim 57, wherein the reaction partner is an antibody and the reactant is an antigen.

60. The method of claim 57, wherein the reaction partner is attached to the luminophore via a covalent bond.

61. The method of claim 57, wherein the reaction partner is attached to the luminophore via a biotin-streptavidin binding interaction.

62. A method for determining the time course of an enzymatic reaction comprising:

(a) forming a composition containing an enzyme, an enzyme substrate and a luminophore, wherein

i) the enzyme catalyzes the reaction of the substrate to form a reaction product;

ii) the luminophore is capable of being induced to electrochemiluminesce; and

iii) the intensity of the ECL signal emitted upon exposure of said composition to electrical energy changes as said reaction progresses; and

(b) exposing the composition to electrical energy and measuring the electrochemiluminescence at different times so as to determine the time course of the reaction.

63. The method of claim 62, wherein the reactant is a cofactor.

64. The method of claim 63, wherein the cofactor is NADH.

65. The method of claim 62, wherein the product is a cofactor.

66. The method of claim 65, wherein the cofactor is NADH.

67. The method of claim 62, wherein the luminophore comprises an organometallic luminophore.

68. A method for determining the time course of a reaction comprising:

(a) forming a composition containing a reactant and a reaction partner of the reactant, wherein the reactant

reacts with the reaction partner to form a reaction product; and

(b) exposing the composition to electrical energy and measuring the electrochemiluminescence at different times.

69. The method of claim 66, wherein said composition further comprises a luminophore.

70. The method of claim 67, wherein said luminophore participates with the reactant, the reaction partner, or the reaction product, to emit electrochemiluminescence upon exposure to electrical energy.

Whitman Breed Abbott & Morgan LLP
File No. KM39091-70

DECLARATION AND POWER OF ATTORNEY

I, a below named inventor, declare that:

My citizenship, residence and Post Office Address are as stated below next to my name;

I believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: **RATE MEASUREMENTS OF BIOMOLECULAR REACTIONS USING ELECTROCHEMILUMINESCENCE**, the specification of which is attached hereto;

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

This is an application to reissue my U.S. Patent 5,527,710, granted June 18, 1996. Applicants verily believe that such patent is wholly or partially inoperative or invalid by reason of the patentee claiming less than he had a right to claim in the patent. Specifically, the claims are insufficient in that they totally fail to claim the method for determining the time course of a reaction and method for determining concentration of a reactant set forth in the new claims added in this reissue application. The new claims do not recite steps (c) - (k) of Claim 1 or limit the claims to a specific type of reaction.

A copy of the claims of my U.S. Patent No. 5,527,710 is attached. The language of each independent claim describing features for which there is no counterpart in the broadest of the new claim 37 in that reissue application is underlined.

The error that was made which is relied on as a basis for this reissue application was the error of failing to realize that applicants had disclosed but not claimed the invention set forth in the accompanying new claims, and instead claimed only a different invention which also was disclosed in the same patent application.

Applicants realized and recognized their error upon reviewing the claims granted in the patent which were found to be limited to one embodiment of the invention rather than properly covering the what applicant had a right to claim.

This error occurred because we were concentrating so intently on the proper protection of the other invention to which the claims of our patent were directed that we simply overlooked the invention for which claims now are being filed.

The foregoing errors arose without any deceptive intention on our part.

We hereby appoint Barry Evans, Registration No. 22,802, John E. Boyd, Registration No. 38,055, and Whitman Breed Abbott & Morgan LLP or their duly appointed associates, our attorneys, with full power of substitution and revocation to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and specify that all communications

about the application are to be directed to the following correspondence address:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

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